JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Glutathione *S*-Transferases of Italian Ryegrass (*Lolium multiflorum*): Activity toward Some Chemicals, Safener Modulation and Persistence of Atrazine and Fluorodifen in the Shoots

Daniele Del Buono* and Gerardina Ioli

Dipartimento di Scienze Agrarie e Ambientali, Università degli Studi di Perugia, Borgo XX Giugno 72, 06121 Perugia, Italy

ABSTRACT: Many varieties of Italian ryegrass (*Lolium multiflorum*) show resistance to herbicides; while this ability was frequently attributed to alterations in the target sites of the herbicide's action of the plant or to an efficient oxidative metabolism, little attention has been paid to glutathione *S*-transferases (GSTs), which are a family of detoxifying enzymes involved in the inactivation of many toxic compounds. To investigate the role of GSTs, seedlings of Italian ryegrass were treated with four herbicides (atrazine, fenoxaprop-ethyl, fluorodifen, metolachlor) and a safener (fenchlorazol-ethyl). All the treatments were well tolerated by the plant, with very low decreases in terms of fresh weight and length of shoots. Regarding GST activity, the chemicals generally determined significant increases in the above enzyme activity toward the model-substrate CDNB. Therefore, the herbicides most GST inducing and the safener were tested themselves as enzyme substrates: constitutive GST activities toward atrazine, fluorodifen and fenchlorazol-ethyl were found, and, in addition, these activities were significantly induced by the safener. Following these results, a HPLC procedure was standardized in order to investigate the persistence of atrazine and fluorodifen in the seedlings of Italian ryegrass and the effect on this of the safener. It was found that the residual amounts of the two herbicides in the shoots were significantly reduced following the safener treatments.

KEYWORDS: Italian ryegrass, glutathione *S*-transferases, herbicides, safener

INTRODUCTION

Italian ryegrass is an annual weed infesting wheat and barley, which shows resistance to different families of herbicides; for this reason and also for its fast growth, the management of this plant is becoming very problematic.[1] In Italy, Italian ryegrass is widely distributed, though more frequently the infestations occur in the central regions. Regarding its resistance to herbicides, it has been proposed that this is mainly due to target-site resistance, which consists of some mutations of the target sites of the herbicides, such as ALS or ACCase.[2,3]

Another very common mechanism of plant resistance to herbicides is nontarget herbicide resistance. This kind of resistance takes into account phenomena of multiple resistance, in which a plant is resistant to herbicides of diverse chemical families, which have different modes of action. The above mechanism is based on a sophisticated detoxification system, in which many enzymes cooperate to inactivate the herbicides, by acting directly on these non-natural substrates.[4] In particular, such detoxification can by divided into three phases: in the first phase the herbicide is hydrolyzed or reduced or oxidized to give a metabolite or breakdown product that is more polar and less mobile.[5] In the second phase, the metabolite is conjugated with endogenous molecules to give a hydrophilic and nontoxic compound, which generally is transferred into the vacuole.[6] Otherwise, in a third phase the metabolites can undergo a further reaction with lignin or cellulose to give an immobilized residue.[7] Different rates of the above enzyme activities are considered responsible for the differences in herbicide resistance among some plants.[8] In general, the participation in nontarget herbicide resistance has been mainly attributed to cytochrome P-450, glutathione *S*-transferase, glycolsyltransferases and ABC transporter proteins.

In this regard, in Italian ryegrass has been evidenced an efficient oxidative inactivation of the xenobiotic substrates, promoted by cytochrome P-450 monoxygenases,[9] while very little attention has been directed to another well-established family of enzymes responsible for the nontarget herbicide resistance: the glutathione *S*-transferases (GST, EC 2.5.1.18). In fact, these multifunctional enzymes can inactivate toxic compounds, such as some herbicides, by conjugating them with glutathione, or they can act as peroxidases in reducing some dangerous byproduct of oxidative stress.[10−12] Besides, the members of the GST family can also be involved in reactions of isomerization or to accomplish other noncatalytic functions.[13] However, the most important role of GSTs is their involvement in the detoxification of exogenous and endogenous toxic compounds; in particular, it has been ascertained that the resistance of some plants depends closely on the activity of these proteins and on the number of isoenzymes of GST that a plant possesses.[14] The GST-mediated herbicide resistance, for the nature of the catalyzed reaction which can have a wide range of substrates, can also be responsible for multiple herbicide resistance (MHR).[15]

Other indications of the role of GSTs in the nontarget herbicide resistance came from the application of safeners.[15,16] These synthetic compounds have been developed to increase the tolerance of cereal crops to herbicides, and in numerous cases it has been demonstrated that the increases of plant resistance to

Received: August 23, 2010
Accepted: January 3, 2011
Published: February 2, 2011

dx.doi.org/10.1021/jf1043713 | *J. Agric. Food Chem.* 2011, 59, 1324–1329

herbicides were due to the induction of GST activity caused by the safeners.[16−19] The great responsiveness of GSTs to safeners has then facilitated the use of some herbicides, which now are supplied in mixture with the specific safeners.[20] Basically, safeners should have a very limited effect on the detoxification enzymes of weeds, if compared to the relative cereal crops; nonetheless, the number of grasses which show a good responsiveness to safeners is increasing.[21,22]

In this context, the strong resistances of Italian ryegrass to herbicides, jointly with the lack of information regarding its GST activity, make the study of this plant of interest, in order to ascertain the eventual role of GST of Italian ryegrass in the resistance to different herbicides, as well as the modulation exerted by the safeners on this enzyme activity.

Therefore, the aims of the research were as follows:

(i) to determine if the treatments of seedlings of Italian ryegrass, with some herbicides and a safener, determined symptoms of stress in terms of growth and fresh weight;
(ii) to determine if the GSTs of this plant were responsive to some herbicides and a safener, by measuring the above enzyme activity toward the model substrate of GST the 1-chloro-2,4-dinitrobenzene (CDNB);
(iii) to determine if Italian ryegrass possessed constitutive GST activity toward the herbicides most GST(CDNB) inducing, and if this activity could be modulated by the safener;
(iv) to determine the persistence on shoots of Italian ryegrass of the herbicides more active in inducing the GST activities, and the effect of the safener on their persistence.

Atrazine, fenoxaprop-ethyl, fluorodifen and metolachlor were chosen as objects of this study because they are representative of some families of herbicides largely diffused in agriculture, and it is documented that they are inactivated by means of GSTs.[10,23,24] Fenchlorazol-ethyl was chosen because it is a safener used in wheat management, where Italian ryegrass is one of the more competitive weeds.

MATERIALS AND METHODS

Chemicals and Apparatus. Atrazine, fenchlorazol-ethyl, fenoxaprop-ethyl, fluorodifen, metolachlor, glutathione and 1-chloro-2,4-dinitrobenzene were supplied by Sigma Aldrich (St. Louis, MO). Acetone, acetonitrile, ethyl acetate, methanol, *n*-hexane and water were all of analytical grade and were purchased from BDH (Poole, U.K.). All other reagents were of ACS grade.

SPE Florisil cartridges were obtained from Chemtek Analytica (U.K.).

A Perkin-Elmer series 410 HPLC, equipped with an LC 95 UV and a C-18 column (4.6 mm i.d.; 25 cm length), was employed for the HPLC determinations of atrazine and fluorodifen residues.

Plant Material. Seeds of Italian ryegrass (*Lolium multiflorum*, 'Hellen') (40 g) were germinated in plastic pots (0.08 m^2) containing sand quartz, prewashed with a solution of hydrochloric acid (10%, v/v) and sterilized with a solution of NaClO (5%, w/v). Seedlings were grown in the dark at 18 °C (relative humidity 80%). After two days, the seedlings were submitted to day−night conditions (12 h of light at 23 °C, light intensity 150 μmol m^{-2} s^{-1}, and 12 h of darkness at 21 °C) and watered daily. When the seedlings were 7 days old, the pots were divided into six groups: one group was left as the control, and the others were singly treated with atrazine, fenchlorazol-ethyl, fenoxaprop-ethyl, fluorodifen and metolachlor, to the recommended field rates. Shoots were collected at 24, 48, and 72 h after the treatments, rinsed with water to remove nonadsorbed chemicals, and dried by blotting, and they underwent determinations of length of shoots and weight and the procedures for GST purification and activity determinations.

GST Extraction and Purification. The extraction of GST was carried out between 0 and 4 °C to avoid protein degradation, and it was performed according to Cummins et al.[25] Shoots of Italian ryegrass (4.0 g) were ground to a fine powder in liquid nitrogen. The powder was suspended in an extraction buffer (1/5, w/v) composed of 100 mM Tris-HCl (pH 7.5), containing 2 mM EDTA, 1 mM dithiothreitol and 1.5% (w/v) polyvinylpolypyrrolidone. After filtration, the homogenate was centrifuged at 10000*g* for 20 min and the supernatant adjusted to 80% saturation with respect to $(NH_4)_2SO_4$, and maintained for 3 h at 4 °C. The resulting suspension was centrifuged at 10000*g* for 10 min, and the pellet was then dissolved in 20 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol and desalted onto a Sephadex G-25.

The protein content of each sample was determined according to Bradford.[26]

Assays of GST(CDNB) Activity. A spectrophotometric procedure was used in order to determine the GST activity toward the CDNB.[22] Briefly, 25 μL of 40 mM CDNB was added to a solution containing 900 μL of 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 6.5), 25 μL of protein extract and 50 μL of 0.1 M GSH (pH 7.0). The amount of conjugate formed by reaction between GSH and CDNB was determined at 340 nm and 35 °C. From this result the amount of conjugate formed in a reaction mixture without the enzymatic extract was then subtracted. The GST activity was expressed in nmol of GSH-CDNB formed s^{-1} mg of $protein^{-1}$.

Assay of GST Activity toward the Herbicides as Substrates. The GST activities toward atrazine, fenchlorazol-ethyl and fluorodifen were determined according to Hatton et al.[27] Each herbicide (2.0 mM; 25 μL), dissolved in acetone, was added to 325 μL of 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 6.5), 50 μL of 10 mM glutathione (pH 7.0) and 50 μL of enzymatic extract. The mixture was incubated at 35 °C for 2 h, and the reaction was stopped by adding 10 μL of 3.6 M HCl. The solution was centrifuged at 5000*g* for 2 min and then frozen at −20 °C. The samples (20 μL) were then analyzed by HPLC to quantify the enzymatic activities. The GST activity was expressed as nmol of herbicide consumed h^{-1} mg^{-1} of protein employed for the assay.

Determination of Atrazine and Fluorodifen Residues. Italian ryegrass seeds were germinated as described above. When the seedlings were 7 days old, the pots were divided into five groups: one group was left as control, and the others were treated with atrazine and fluorodifen in combination or not with the safener fenchlorazol-ethyl. The treatments were performed to the recommended field rates, and samples of shoots (2.0 g) were collected at 24, 48, 72, 96, and 144 h after the treatments. The amount of herbicide residues in the shoots was determined according to Del Buono et al.[28] Briefly, the samples were powdered in liquid nitrogen and then extracted with methanol (w/v, 1:5); the resulting suspension was filtered, dried under vacuum and rinsed with 2 mL of *n*-hexane. The solution was then charged on a SPE cartridge Florisil column (1000 mg/6 mL, 170 μm, 80 A), preactivated with 15 mL of *n*-hexane, washed with 5 mL of *n*-hexane and then recovered with 4 mL of a solution composed by ethyl acetate/*n*-hexane (v/v, 2:3). The recovered fractions were evaporated to dryness, rinsed with 1 mL of methanol and subjected to HPLC analyses.

For the HPLC determination of residues, the injection volume was 20 μL. For atrazine, the HPLC separation was done using 60% of acetonitrile and 40% of water, at flow rate of 0.7 mL min^{-1} and the wavelength of 220 nm. Under these conditions the retention time was 9 min. For fluorodifen, the HPLC conditions were 75% of acetonitrile and 25% of water, flow rate of 0.8 mL min^{-1} and wavelength at 265 nm. Under these conditions the retention time was 10 min.

To validate the method some recovery tests were performed, by adding to the samples of shoots (2.0 g) adequate amounts of herbicides in order to give the concentrations of 0.2, 0.5, 2.0, and 10.0 mg kg^{-1}.

Table 1. Length of Shoots of Italian Ryegrass Treated with the Herbicides, Compared with Untreated Samples[a]

	length of the shoots (cm)		
	24 h	48 h	72 h
control	10.0 a	10.5 a	12.4 a
atrazine	8.6 b	9.2 b	10.7 b
fenclorazol-ethyl	9.5 a	9.6 a	10.5 b
fenoxaprop-ethyl	9.6 a	9.8 a	12.9 a
fluorodifen	10.5 a	10.6 a	11.9 a
metolachlor	10.3 a	10.5 a	10.5 b

[a] The data represent the means of 30 determinations. Means within the same column followed by the same letter are not significantly different from the control at a 5% level using the *t* test of Student.

Table 2. Fresh Weight of Shoots of Italian Ryegrass Treated with the Herbicides, Compared with Untreated Samples[a]

	fresh weight of 10 shoots (g)		
	24 h	48 h	72 h
control	0.146 a	0.155 a	0.168 a
atrazine	0.132 b	0.136 b	0.146 b
fenclorazol-ethyl	0.140 a	0.141 b	0.153 b
fenoxaprop-ethyl	0.141 a	0.156 a	0.175 a
fluorodifen	0.156 a	0.155 a	0.183 a
metolachlor	0.149 a	0.156 a	0.163 b

[a] The data represent the means of six determinations. Means within the same column followed by the same letter are not significantly different from the control at a 5% level using the *t* test of Student.

RESULTS

Symptoms of Stress. In order to verify eventual symptoms of stress, the length of shoots and the fresh weight were determined for the samples treated, at the respective field rates, with atrazine, fenoxaprop-ethyl, fluorodifen, metolachlor and with the safener fenchlorazol-ethyl, and collected at 24, 48, and 72 h after the treatments (Tables 1 and 2). In general, it was ascertained that the treatments with atrazine, metolachlor and fenchlorazol-ethyl resulted in decreases of length of shoots (Table 1). In particular, atrazine decreased the length of shoots at 24, 48, and 72 h by 14.0, 12.4 and 13.7%, respectively. Metolachlor and fenchlorazol-ethyl caused a slight decrease in length of shoots, which was for both the chemicals of 15.3% at 72 h after the treatments. On the other hand, fenoxaprop-ethyl and fluorodifen did not cause any decreases in the length of shoots.

Regarding the fresh weight, atrazine, metolachlor and fenchlorazol-ethyl determined some decreases in the above parameter (Table 2). In fact, it was found that atrazine reduced the fresh weight by 9.6, 12.3 and 13.1%, at 24, 48, and 72 h, respectively. Fenchlorazol-ethyl reduced the fresh weight at 48 and 72 h after the treatments, and the decreases were 9.0% and 8.9%, respectively. Metolachlor slightly decreased the fresh weight only at 72 h after the treatments. The treatments with fenoxaprop-ethyl and fluorodifen did not caused decreases in fresh weight.

GST(CDNB) Activity. The GST activity for the treated and untreated samples was assayed on the model substrate CDNB over a time period of 72 h. In particular, fluorodifen strongly increased the GST(CDNB) at 24, 48, and 72 h after the treatments, and the inductions were 98, 371 and 30%, respectively. Atrazine induced the GST activity at 24, 48, and 72 h after the treatment of 33, 104 and 25%. Also fenoxaprop-ethyl had effect on the GST(CDNB) activity at 24, 48, and 72 h after the treatments, and it determined increases in enzyme activity of 52, 20 and 23%, respectively. On the other hand, metolachlor did not show any effect at 24 and 48 h after the treatment, while it determined a decrease on GST activity at 72 h after the treatments of 26%. Regarding the safener fenchlorazol-ethyl, it caused significant increases of activity at 24, 48, and 72 h after the treatment and they were of 19, 197 and 133%, respectively.

Therefore, among the five chemicals tested, three caused very strong inductions of GST(CDNB) activity, while the other two caused more modest enzyme inductions or no effects. In particular, the highest effects on GST(CDNB) activity, during the all experimental period, were caused by the chemicals in the following decreasing order: fluorodifen, fenchlorazol-ethyl and atrazine. In addition, all these chemicals caused the highest inductions of enzyme activities at 48 h after the treatments (Table 3).

Table 3. GST(CDNB) Activity at 24, 48, and 72 h after the Treatments, Compared with Untreated Samples[a]

	GST(CDNB) (nmol s^{-1} mg of $protein^{-1}$)		
	24 h	48 h	72 h
control	0.340 a	0.406 a	0.373 a
atrazine	0.452 b	0.828 b	0.466 b
fenclorazol-ethyl	0.404 b	1.205 b	0.869 b
fenoxaprop-ethyl	0.518 b	0.489 b	0.459 b
fluorodifen	0.673 b	1.914 b	0.486 b
metolachlor	0.300 a	0.390 a	0.277 c

[a] The data represent the means of triplicate determinations. Means within the same column followed by the same letter are not significantly different from the control at a 5% level using the *t* test of Student.

GST Activity toward the Herbicides in Unsafened and Fenchlorazol-ethyl Safened Shoots of Italian Ryegrass. Following the data obtained from the investigations on GST activity toward CDNB, the constitutive GST activities have been determined toward atrazine, fluorodifen and fenchlorazol-ethyl, for protein extracted from untreated shoots of Italian ryegrass. This was done to evaluate if Italian ryegrass possessed constitutive GST activity to inactivate these herbicides by means of conjugation with glutathione. The three chemicals were chosen because of their great effectiveness in inducing the GST(CDNB) activity during all the experimental period of 72 h, with a maximum effect at 48 h after the treatments. Italian ryegrass showed constitutive GST activity toward atrazine, fluorodifen and fenchlorazol-ethyl (Table 4). The highest activity was found toward fluorodifen, followed by fenchlorazol-ethyl and, finally, by atrazine (Table 4). After these experiments, seedlings of Italian ryegrass were treated with the safener fenchlorazol-ethyl, in order to ascertain if the safener could induce constitutive GST activities toward atrazine, fluorodifen and itself. Therefore, the safened shoots were collected 48 h after the treatment, because it was the time of maximum safener induction of GST(CDNB) activity. The protein extracts were then submitted to the determination of GST activities and, as reported in Table 4, it was found that the safener significantly increased the GST activity toward atrazine

Table 4. Extractable GST Activities toward Atrazine, Fluorodifen and Fenchlorazol-ethyl from Shoots of Untreated and Fenchlorazol-ethyl Treated Italian Ryegrass[a]

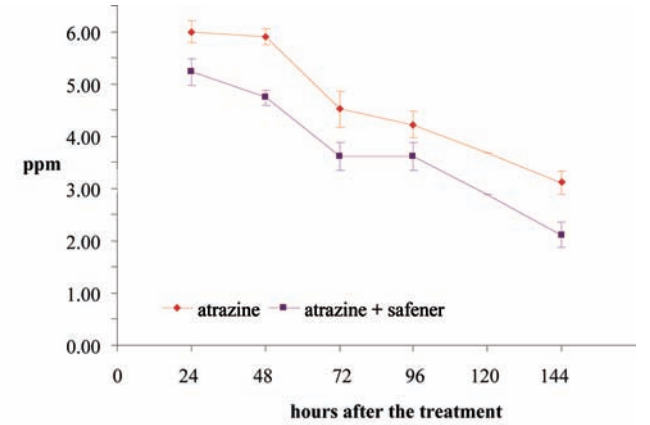
	GST(herbicide) (nmol h^{-1} mg of protein^{-1})		
	atrazine	fluorodifen	fenchlorazol-ethyl
control	3.5 a	36.7 a	29.8 a
fenclorazol-ethyl	4.6 b	45.1 b	33.0 b

[a] The data represent the means of triplicate determinations. Means within the same column followed by the same letter are not significantly different from the control at a 5% level using the *t* test of Student.

and fluorodifen. In the case of atrazine the induction was 31%, while in the case of fluorodifen the increase was 23%, with respect to the unsafened samples. The safener caused also an increase in the GST activity toward itself (Table 4).

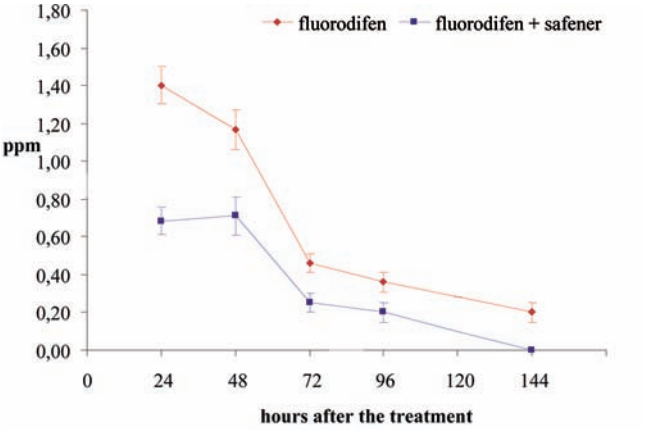
Accumulation and Persistence of Atrazine and Fluorodifen in the Unsafened and Fenchlorazol-ethyl Safened Shoots of Italian Ryegrass. The method based on a HPLC procedure, employed in order to determine the residues of atrazine and fluorodifen, was validated by means of recovery tests performed at the concentrations of the two chemicals of 0.2, 0.5, 2.0, and 10.0 mg kg^{-1}. The percentages of recovery for the two compounds, at these four fortification levels, ranged from 85% to 90% for atrazine and from 95% to 100% for fluorodifen. The limits of quantitation (LOQ), defined as the amount at twice the signal/noise ratio,[29] were 1.0 and 4.0 ng for atrazine and fluorodifen, respectively. The linearity among the different concentrations of the two herbicides and the chromatographic areas of the peaks were investigated at the concentrations of 0.1, 0.5, 1.0, 2.0, 10, and 20 μg mL^{-1}. This study evidenced that the calibration curves were characterized by average correlation coefficients (r^2) of 0.999. These data indicated a good linearity of the responses, and, if jointly considered with the data of recovery tests, these results make this method adequate in order to study the persistence of the two herbicides.

The curves of Figure 1 and Figure 2 show significant effects of the safener on the persistence of both atrazine and fluorodifen. In fact, safened samples showed lower amounts of residues, and in the case of fluorodifen they were also lesser persistent, with respect to the residues found in unsafened samples. In particular, the residues of atrazine reached the highest accumulations at 24 h after the treatments, and they were 6.00 ppm and 5.23 ppm, in the unsafened and safened shoots, respectively. Residues of atrazine were found during all the experimental period investigated, and the lowest amount of residues was determined at 144 h after the treatments; in fact, they were 3.11 and 2.10 ppm in unsafened and safened samples, respectively. Overall, the average content of atrazine, observed over a time of 144 h, was 4.75 ppm in unsafened samples, while the average content of herbicide residues determined in the safened samples was 3.86 ppm. Therefore, fenchlorazol-ethyl caused an average decrease of atrazine residues of 19%. With regard to the fluorodifen residues, they reached the highest values of accumulations at 24 h after the treatments. In fact, 1.40 and 0.68 ppm of fluorodifen for the unsafened and safened shoots of Italian ryegrass, respectively, were found. The persistence of fluorodifen in unsafened samples covered all the experimental period of 144 h, while in the safened shoots it was not detectable at 144 h after the treatments. Overall, the average content of fluorodifen, observed over a time of 144 h, was 0.72 ppm in unsafened samples; the average content of herbicide residues determined in the safened samples, until 96 h after the treatments, was 0.46 ppm. Therefore, fenchlorazol-ethyl caused an average residue decrease of fluorodifen of 36%.



	atrazine	*atrazine with safener*	*decrease (%)*
0	-	-	-
24 h	6.00a	5.23b	12.8
48 h	5.90a	4.74b	19.7
72 h	4.52a	3.62b	19.9
96 h	4.22a	3.61b	14.4
144 h	3.11a	2.10b	32.5

Figure 1. Residues of atrazine in shoots of Italian ryegrass treated with atrazine and with a mixture composed of atrazine and fenchlorazol-ethyl. The data represent the means of triplicate determinations. Means within the same column followed by the same letter are not significantly different from the control at a 5% level using the *t* test of Student.



	fluorodifen	*fluorodifen with safener*	*decrease (%)*
0	-	-	-
24 h	1.40a	0.68b	51.4
48 h	1.17a	0.71b	32.3
72 h	0.46a	0.25b	45.7
96 h	0.36a	0.20b	45.5
144 h	0.20a	-	-

Figure 2. Residues of fluorodifen in shoots of Italian ryegrass treated with fluorodifen and with a mixture composed of fluorodifen and fenchlorazol-ethyl. The data represent the means of triplicate determinations. Means within the same column followed by the same letter are not significantly different from the control at a 5% level using the *t* test of Student.

DISCUSSION

Data of length of shoots and fresh weights are in accordance with the documented tolerance of Italian ryegrass to herbicides.[30,31] The interferences on these parameters were of scarce relevance: in fact, very modest decreases in the length of the shoots and in the fresh weight have been ascertained in response to fenchlorazol-ethyl and metolachlor, while only atrazine exerted a depressing effect for all the experimental period.

Regarding GST(CDNB) activity, our findings show that the GSTs are differentially induced by the treatments with the herbicides and the safener. In fact, atrazine, fluorodifen and fenchlorazol-ethyl caused the strongest increases in GST activity. In the case of fenoxaprop-ethyl, which belongs to class of aryloxyphenoxypropionates, a significant activation in the above enzyme activity took place, although to a lesser extent. In every case, the highest inductions in the GST(CDNB) activities occurred at 48 h after treatment for atrazine, fluorodifen and fenchlorazol-ethyl.

The GST activity of shoots of Italian ryegrass was deeply investigated by determining the constitutive activity toward the herbicides most GST(CDNB) inducing, and toward the safener fenchlorazol-ethyl. In particular, it was ascertained that the protein extracts possessed significant constitutive GST activity toward atrazine, fenchlorazol-ethyl and fluorodifen. It is important to remark as the constitutive GST activity toward fluorodifen was significantly higher than that determined toward atrazine. These differences in the rates of conjugation of the herbicides are in accordance with the lower persistence and accumulation determined for fluorodifen with respect to atrazine (see below). Following these data, the effect of the safener on the GST activities toward these chemicals was investigated. The results showed that the GST activity toward fluorodifen and atrazine was significantly increased by the safener treatments. This modulation of the GST activity has been observed also for other plants treated with safeners, confirming the importance of the involvement of GSTs in the detoxification of some xenobiotics.[12,21,26]

The wide spectrum of conjugative activity of GSTs toward the different chemicals tested is attributable to the structural characteristics of these enzymes. In fact, it is known that they are dimeric proteins, composed of different subunits; each subunit has an independent active site, consisting of two components: a glutathione binding site and a second site which binds xenobiotic hydrophobic substrates;[32] from this it is explainable the different activities exhibited by Italian ryegrass against CDNB, atrazine, fluorodifen and fenchlorazol-ethyl, as well as the safener specificity in the induction of GST activity.

The consequence of the fenchlorazol-ethyl activation of GSTs of Italian ryegrass is shown by the data on the accumulation and persistence of atrazine and fluorodifen in the unsafened and fenchlorazol-ethyl safened shoots (Figures 1 and 2). The persistence of atrazine and fluorodifen was significantly reduced in safened shoots, and already at 24 h after the treatment there were marked differences in the amount of residues of herbicides between safened and unsafened samples. The reduced persistence and accumulation determined in safened samples are in accordance with the safener induction of the GST activities (Table 4).

Finally, the research evidenced the presence of active naturally expressed glutathione *S*-transferases in Italian ryegrass. These GSTs were active on some chemicals, and the safener treatment determined a more effective detoxification of two herbicides, as evidenced also by the curves of persistence. These results are in accordance with studies performed on other graminaceous plants, which showed that the GST activity permitted the plants to detoxify some herbicides.[10,22,23,33]

In conclusion, this research showed the capacity of glutathione *S*-transferases of Italian ryegrass to act on some herbicides, so helping to elucidate the strong resistance exhibited by this plant. The activity of GSTs of Italian ryegrass should be considered as an integral and prominent factor to be jointly considered to the traditional ways of resistance of this plant so far studied.

Moreover, GSTs have a very important role in the resistance of plants to diverse classes of graminicide herbicides that have different modes of action. In particular, multiple herbicide resistance (MHR) has been associated with wild grasses which display resistance to graminicide herbicides, with differing modes of action; MHR is problematic, and its wide spread around the world is causing increasing losses in crop yield.[15]

On the other hand, although Italian ryegrass is a not cultivated species, its detoxifying activities toward some herbicides and their responsiveness to fenchlorazol-ethyl may assume relevance in relation to practices of soil−water protection. In fact, the detoxificative abilities of Italian ryegrass make the plant suitable to vegetate buffer strips, which are uncultivated zones along the boundaries of noncompeting crops.[34] The function of these zones is to reduce the environmental contamination caused by the surface runoff of various pollutants, herbicides included, and prevent loss of sediments.[35] Moreover, the combination of a plant, with decontaminant abilities, and of an adequate safener could be considered as a tool to potentiate these buffer strips. From this point of view, plants could permit also the recovery of polluted sites, in order to give back these areas to agriculture or to the community.

AUTHOR INFORMATION

Corresponding Author

*Tel: +39 075 585 6225. Fax: +39 075 585 6239. E-mail: delbuono@unipg.it.

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